Mechanism of Inactivation of Erythrocyte Membrane Adenosine Triphosphatase by Carbodiimides*

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ABSTRACT: In order to investigate the role of carboxyl groups in the adenosine triphosphatase (ATPase) activity of the erythrocyte membrane, hemoglobin-free ghosts were treated with a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC). Progressive inactivation of ATPase activity was observed, with the Mg^{2+}-ATPase exhibiting greater susceptibility to inactivation compared to the Na^+,K^+-stimulated ATPase. Evidence was obtained implicating membrane carboxyls in the inactivation process. Addition of nucleophiles simultaneously with EDAC afforded protection against enzyme inactivation. This observation suggested that EDAC-activated carboxyls might undergo nucleophilic attack by some proximal membrane nucleophile, thereby giving rise to an intramolecular cross-link, most probably a peptide bond. Although ATP afforded protection against ATPase inhibition by EDAC, nonsubstrate nucleotide triphosphates exhibited the same behavior. These nucleotide effects were most probably the result of alterations in membrane conformation rather than interaction at the level of the catalytic site of ATPase. The membrane modification by carbodiimide appeared to be a relatively specific one, since two other membrane enzymes (DPNase- and K^+-independent *p*-nitrophenyl phosphatase) were unaffected. Carbodiimide modification did, however, lead to inhibition of the K^+-dependent *p*-nitrophenyl phosphatase activity of the membrane, and this finding was consistent with the hypothesis that this enzyme bears some functional relationship to ATPase. Studies using EDAC and [^{14}C]glycine ethyl ester indicated that nucleophile incorporation was confined to the lipid-free component of the membrane and did not appear to involve the carboxyls of phosphatidylserine or sialic acid. The extent of labeling of the protein fraction indicated that only 10% of the total membrane protein carboxyls were susceptible to EDAC–nucleophile attack. Modification of only a small proportion of these available carboxyls was sufficient for complete inactivation of ATPase. It is therefore proposed that carbodiimide attack on the erythrocyte membrane gives rise to disruption of the ATP-hydrolyzing system *via* a selective structural perturbation of the membrane. These studies offer an interpretation for the effects of carbodiimides on other membrane systems involved in energy transduction processes.

Despite the widespread occurrence of ATP-hydrolyzing enzymes and the implication of ATPase activity in a number of fundamental physiological processes including muscular contraction (Wu, 1969), active cation transport (Glynn, 1968), and electron transport (Bulos and Racker, 1968), understanding of the molecular features responsible for the catalytic process and its regulation remains incomplete.

In the erythrocyte membrane two general types of ATPase activities have been recognized: one being maximally stimulated in the presence of magnesium alone (Mg^{2+}-ATPase) and the other exhibiting maximal activity in the presence of sodium and potassium in addition to magnesium (Na^+,K^+-ATPase). Although the function of erythrocyte Mg^{2+}-ATPase is unknown, involvement of the Na^+,K^+-ATPase system in active transport of sodium and potassium across the erythrocyte membrane appears to be firmly established (Dunham and Glynn, 1961). Membrane integrity and Na^+,K^+-ATPase activity seem to be closely interrelated (Albers, 1967), and Na^+,K^+-ATPase might represent a link between enzymatic and structural aspects characteristic of the ion transport process in the erythrocyte membrane.

The hydrolysis of ATP by Na^+,K^+-ATPase is believed to proceed *via* a Na^+-dependent phosphorylation followed by a K^+-dependent dephosphorylation of the phosphorylated intermediate (Albers, 1967). Studies on the characterization of the phosphorylated intermediate have provided evidence favoring a protein carboxyl group as the acceptor of the terminal phosphate of ATP during cation transport (Hokin *et al.*, 1965).

Carbodiimides, which are well-known carboxyl group activating reagents (Kurzer and Douraghi-Zadeh, 1967), have been shown to inhibit an ATPase located on the inner mitochondrial membrane (Bulos and Racker, 1968) and also a membrane-bound ATPase in *Streptococcus faecalis* (Abrams *et al.*, 1969). Studies thus far have not elucidated the chemical mechanism involved in the inhibition, and it seemed particularly important to determine if the carboxyl group postulated to be involved as the acyl-phosphate intermediate during ATP hydrolysis could be the site of action of this class of inhibitor. It was therefore decided to utilize carbodiimide modification as a probe to investigate the role of erythrocyte membrane carboxyl groups in the ATPase activity of the membrane.

Materials

Tris-ATP, hydroxylamine hydrochloride, 2,4-dinitrofluorobenzene, glycine methyl ester hydrochloride, *p*-hydroxymercuriphenylsulfonic acid (monosodium salt), and neura-

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minidase type V were all obtained from Sigma Chemical Co., St. Louis, Mo.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC)[1] was obtained from the Ott Chemical Co., Muskegon, Mich.

2-Ethoxy-*N*-carbethoxy-1,2-dihydroquinoline (ECDQ) was a generous gift of Professor B. Belleau, Department of Chemistry, University of Ottawa, Can.

Tyrosine and histidine methyl esters were obtained from Mann Research Laboratories, Inc.

[^{14}C]Glycine ethyl ester hydrochloride (specific activity 3 mCi/mmole) was obtained from the New England Nuclear Corp.

Methods

Preparation of Erythrocyte Membranes. Human erythrocyte membranes were prepared from outdated (21-day old) blood stored in acid–citrate–dextrose by the technique of stepwise osmotic lysis. The method was as previously described (Schrier, 1967) except that in the final step, 0.01 M Tris (pH 7.5) replaced isotonic NaCl. The resulting preparations usually contained 3–4 mg of protein/ml.

Shearing of Erythrocyte Membranes. In order to eliminate permeability effects which might affect the accessibility of inhibitor to a potential site of attack, membranes were sheared in a chilled Aminco French press. Unsheared fragments were removed by centrifugation at 13,000*g* for 10 min at 4° (Schrier *et al.*, 1970).

ATPase Activity Measurement. The method used was a slight modification of that of Post *et al.* (1960). Incubations were carried out in a total volume of 3.0 ml containing 0.5–1.0 mg of membrane protein, 6 μmoles of Tris-ATP, 180 μmoles of imidazole–glycylglycine buffer (pH 7.4), and 9 μmoles of $MgCl_2$. For total ATPase determinations, the incubation mixture also contained 250 μmoles of NaCl and 90 μmoles of KCl; these cations were absent from media for Mg^{2+}-ATPase assay. Samples were incubated for 1 hr at 37°, the reaction was stopped by adding 1.0 ml of 20% cold trichloroacetic acid, and reaction mixtures were centrifuged at 30,000*g* for 5 min. Supernatant solutions were decanted and assayed for P_i by the Fiske–Subbarow (1925) method. The protein content of the membrane material was determined by the Lowry method (Lowry *et al.*, 1951) using five-times-recrystallized bovine serum albumin (Armour) as standard. ATPase specific activities were expressed as micromoles of P_i per milligram of membrane protein per hour, and Na^+,K^+-ATPase activity was the difference between total and Mg^{2+}-ATPase activities. $K_{m,app}$ and V_{max} total values were determined by the double-reciprocal plot of Lineweaver–Burk (1934).

p-Nitrophenyl Phosphatase Activity. The method of Fujita *et al.* (1966) was extensively modified so that assay conditions (preincubation time, protein concentration, pH, etc.) could approximate as closely as possible those of the ATPase assay. In this regard, it was convenient to choose a substrate concentration giving approximately half-saturation. Nevertheless, the resulting specific activities of the K^+-dependent *p*-nitrophenyl phosphatase were equal or greater than those calculated from the data of Pouchan *et al.* (1969) who used 6.3 mM *p*-nitrophenyl phosphate. The 3.0-ml reaction mixture contained 5 μmoles of *p*-nitrophenyl phosphate, 5 μmoles of $MgCl_2$, and 195 μmoles of imidazole–glycylglycine buffer (pH 7.4). Incubations were performed at 37° for 1 hr. For total phosphatase activity measurement the reaction mixture contained 90 μmoles of KCl. The supernatant solution following centrifugation was made alkaline with 20% KOH, and the concentration of *p*-nitrophenol was determined from the absorbance at 412 mμ. Specific activities were calculated as for ATPase, and K^+-dependent phosphatase represented the difference in activities between reaction mixtures with and without potassium.

DPNase Activity. The method used is based on the formation of a complex between residual DPN and cyanide ion (Kaplan, 1955).

Inhibition Studies. All inhibitors were preincubated with the erythrocyte membranes in a 1.0-ml volume, and at the end of the preincubation the ATPase reaction was initiated by the addition of 2.0 ml of a mixture containing ATP, buffer, $MgCl_2$ with or without NaCl, and KCl. Specific activities were determined as described above, and the results were expressed as per cent inhibition relative to an appropriate control incubated in the absence of inhibitor. K_i values were evaluated by plotting the reciprocal of the specific activity on the ordinate *vs.* the inhibitor concentration as the abscissa. Hill plots were performed as described by Loftfield and Eigner (1969). Identical conditions were used for *p*-nitrophenyl phosphatase inhibition studies, namely, preincubation in a 1.0-ml volume, with a final incubation mixture of 3.0 ml.

Labeling of Membranes with [^{14}C]Glycine Ethyl Ester. In a typical experiment, 1.0-ml membrane preparation containing 3–4 mg of protein, 1.0 ml of H_2O, and 0.010 ml of [^{14}C]glycine ethyl ester (50 mCi/ml) were incubated at room temperature for 15 min after which 0.1 ml of 0.02 M EDAC solution was added, and the mixture was incubated at room temperature for 15–60 min. Isotonic NaCl (40 ml) was added, and the suspension was centrifuged at 30,000*g* for 15 min at 4°. The supernatant solution was discarded, and the residue was resuspended in 40 ml of isotonic NaCl, and washing was repeated three times to ensure complete removal of unreacted water-soluble [^{14}C]glycine ethyl ester. The residue was suspended in a small volume of water and subsequently frozen and lyophilized. The dried residue was weighed and dispersed with sonication in 1 ml of 1% sodium dodecyl sulfate. Bray's counting solution (15 ml) was added, and the radioactivity of ^{14}C was determined in a Packard liquid scintillation spectrometer (counting efficiency 70%).

Lipid Extraction of Radioactively Labeled Membranes. Lyophilized erythrocyte ghosts (5 mg) labeled with EDAC and [^{14}C]glycine ethyl ester were suspended in 10 ml of chloroform–methanol (2:1, v/v) and the mixture was stirred for 0.5 hr at room temperature. The material was centrifuged, the supernatant was retained, and the residue was reextracted with chloroform–methanol. The supernatant solutions were combined, evaporated to dryness, and taken up in 1.0 ml of toluene. Bray's solution (15 ml) was added, and the radioactivity of the sample was determined.

Neuraminidase Treatment of Membranes. Membrane prep-

[1] Abbreviations used are: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; ECDQ, 2-ethoxy-*N*-carbethoxy-1,2-dihydroquinoline.

TABLE I: Time Course of EDAC Inhibition of ATPase in Whole and Sheared Membranes.

Sample	Preincubn Time (min)	Total ATPase[a]	Mg^{2+}-ATPase	Na^+,K^+-ATPase
		Part a		
Whole membranes	2–60[c]	0.578	0.446	0.130
Whole membranes + EDAC[b]	2	0.390 (32)[d]	0.250 (44)	0.140 (0)
	5	0.310 (46)	0.160 (64)	0.150 (0)
	15	0.150 (74)	0.060 (87)	0.090 (32)
	30	0.060 (90)	0.030 (93)	0.030 (77)
	60	0.000 (100)	0.000 (100)	0.000 (100)
		Part b		
Sheared membranes	2–60[c]	0.320	0.200	0.120
Sheared membranes + EDAC[b]	2	0.200 (37)	0.114 (43)	0.086 (28)
	5	0.180 (44)	0.080 (60)	0.100 (17)
	15	0.110 (66)	0.040 (80)	0.070 (42)
	30	0.050 (84)	0.030 (85)	0.020 (84)
	60	0.000 (100)	0.000 (100)	0.000 (100)

[a] ATPase values represent micromoles of phosphate per hour per milligram of membrane protein. [b] Concentration EDAC in preincubation, 2.5×10^{-3} M. [c] Preincubation for control membranes was also varied from 2 to 60 min. Since all values were comparable, the mean values are recorded. [d] Numbers in parentheses represent per cent inhibition relative to the control.

TABLE II: Concentration Dependence of Inhibition of Membrane ATPase by EDAC.[a]

Concn of EDAC (mM)	(a) Whole Membranes % Inhibn			(b) Sheared Membranes % Inhibn		
	Total ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase	Total ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase
0.5	23	42	−80	20	24	−18
1.0	39	65	−100	21	44	−160
1.5	47	65	−40	24	53	−240
2.0	55	75	−50	37	61	−160
2.5	60	71	−20	44	65	−150
K_i (mM)	1.6	0.7		3.4	1.3	

[a] 5-min preincubation at 23°.

aration (1.0 ml), containing 5 mg of protein, 0.05 ml of neuraminidase (1 mg/ml), and 0.1 ml of $CaCl_2$ (0.05 M), was combined in a total volume of 2.0 ml and incubated at 37° for 30 min. The material was centrifuged at 30,000*g* for 15 min, the supernatant was discarded, and the residue was washed by suspending in water and centrifuging (Schrier, 1967).

Results

Carbodiimide Inactivation of Erythrocyte Membrane ATPase. In order to study the role of carboxyl functions in the ATPase system, hemoglobin-free erythrocyte ghosts were preincubated with a water-soluble carbodiimide, EDAC. Progressive inactivation of ATPase activity was observed with complete loss of activity occurring over a period of 60 min at room temperature and an EDAC concentration of 2.5 mM (Table Ia). The foregoing studies were repeated on membranes sheared into microvesicles so that factors affecting accessibility of the inhibitor to its site of action would be minimized. The results are summarized in Table Ib, and it is clear that disruption of gross membrane structure had no effect on the inhibition process.

The greater susceptibility to inhibition of Mg^{2+}-ATPase (as compared to Na^+,K^+-ATPase), which was most apparent in both whole and sheared membranes at low levels of inhibition, was even more clearly revealed when the concentration dependence of EDAC inhibition was studied (Table IIa,b). The apparent stimulation of Na^+,K^+-ATPase activity (indicated as negative inhibition in Table II) on treatment with EDAC was a frequently, but not invariably obtained effect. The effect of the carbodiimide on Na^+,K^+-ATPase appeared to depend in part on the particular membrane preparation as well as the concentration of membrane protein in the pre-

TABLE III: Protection by Nucleophiles against EDAC Inactivation of ATPase.

Nucleophiles[a]	% Protection[b] Total ATPase	% Protection[b] Mg^{2+}-ATPase
H_2O	0	0
Histidine methyl ester	89	85
Tyrosine methyl ester	69	61
Glycine methyl ester	60	54
2-Mercaptoethanol	60	46
Choline chloride	19	8
Hydroxylamine	100	100

[a] At concentration of 2 mM in preincubation mixture. The concentration of EDAC in all tubes was 2 mM; temperature, 23°. [b] Protection = 100 − % inhibition. Value for tubes incubated with H_2O alone was arbitrarily set at 0% protection.

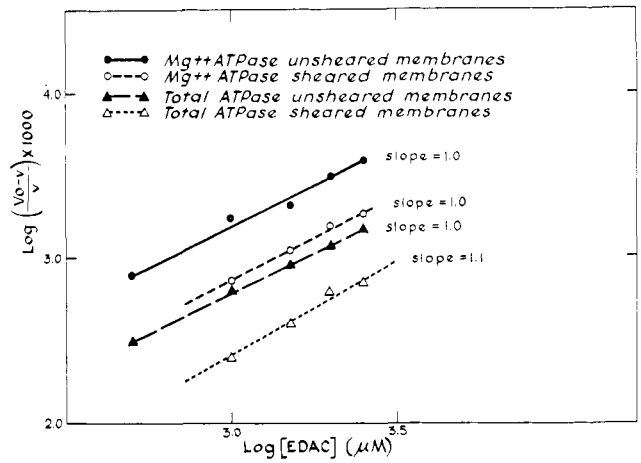

FIGURE 1: Hill plot for EDAC inhibition of ATPase of sheared and unsheared erythrocyte membranes. Log $[(V_0 - v)/v] \times 1000$ *vs.* log concentration EDAC, where V_0 = uninhibited rate and v = inhibited rate.

incubation mixture. Inhibition of the total and Mg^{2+}-ATPase activities of whole membranes was associated with K_i values of 1.6 and 0.7 mM, respectively. For sheared membranes, the corresponding values were 3.4 and 1.3 mM for total and Mg^{2+}-ATPase. The Hill plots for the EDAC inhibition of total and Mg^{2+}-ATPase activities of whole and sheared membranes were all linear and had slopes of 1 (Figure 1). This indicated that cooperativity was not involved in the inactivation process and that a single type of interaction mechanism determines ATPase inhibition by EDAC (Loftfield and Eigner, 1969).

The carbodiimide inactivation of the membrane ATPase could not be reversed by repeated washings of the ghosts with water, or with a solution of 100 mM NaCl and 30 mM KCl, or 3 mM Mg^{2+} in addition to NaCl and KCl. These washing experiments also indicated that the inhibitory effects of EDAC were not due to interaction with ATP or other components of the assay medium, because in these studies the washing served to remove unbound EDAC prior to the addition of substrate and cofactors.

Mechanism of Carbodiimide Inhibition. ROLE OF MEMBRANE CARBOXYL GROUPS. The increasing EDAC inactivation of ATPase with time and the carboxyl-activating properties of the carbodiimide moiety suggested that the inhibition process might involve covalent bond formation at membrane carboxyl sites. Carbodiimides are known to react with carboxyl groups with the formation of an anhydride-like adduct which is very susceptible to nucleophilic attack (Kurzer and Douraghi-Zadeh, 1967). If the mechanism of ATPase inactivation involved cross-link formation between a carbodiimide-activated carboxyl and a proximal membrane nucleophile, it was proposed that addition of a nucleophile to the medium might decrease the level of inhibition by competing with the membrane nucleophile for attack on the reactive EDAC–carboxyl adduct thereby decreasing the extent of cross-linking.

Hydroxylamine, amino acid methyl esters, and mercaptoethanol when present simultaneously with EDAC (in equimolar amounts) all diminished the inhibitory effects of the carbodiimide on total and Mg^{2+}-ATPase activities (Table III). In contrast to this behavior, when hydroxylamine (2 mM) was added subsequent to EDAC attack, no regeneration of enzyme activity could be detected, even after a 60-min incubation at room temperature. Therefore, while hydroxylamine could protect against EDAC inactivation of ATPase, the already inhibited enzyme was insensitive to hydroxylamine reversal under the conditions used.

Although carbodiimides are generally regarded as carboxyl group modifiers, other groups such as aliphatic and phenolic hydroxyls and amino groups may undergo reaction (Riehm and Scheraga, 1966; Carraway and Koshland, 1968). In order to ascertain whether a carboxyl function was involved in the inactivation, the effects of EDAC were compared to those of ECDQ, a highly specific reagent for the activation and modification of carboxyl groups under the present conditions (Belleau *et al.*, 1968; Belleau and Maleck, 1968). ECDQ was found to inhibit ATPase activity, with a marked selectivity for the Mg^{2+}-ATPase component (Table IV). The K_i for Mg^{2+}-ATPase inhibition was 0.25 mM, and a

TABLE IV: Inhibition of Erythrocyte Membrane ATPase by ECDQ.

Concn of ECDQ in Preincubn[a] (mM)	% Inhibition Total ATPase	% Inhibition Mg^{2+}-ATPase	% Inhibition Na^+,K^+-ATPase
0	0	0	0
0.009	5	2	+9
0.037	11	12	+9
0.075	8	21	−9
0.150	7	43	−37
0.300	30	69	−18
0.610	55	86	+18

[a] 15-min preincubation at 23°.

TABLE V: Effect of Sulfhydryl Inhibition on EDAC Inactivation of ATPase (μmole of Phosphate/mg of Protein per hr).

Preincubn[a] Time (min)	Sample	Total ATPase	Mg^{2+}-ATPase	Δ (Control − Inhibited Sample) Total ATPase	Δ (Control − Inhibited Sample) Mg^{2+}-ATPase
	Control	0.80	0.55		
5	PHMPS[b]	0.64	0.44	0.16	0.11
10	PHMPS[b]	0.69	0.44	0.11	0.11
5	EDAC[c]	0.57	0.25	0.23	0.30
10	EDAC[c]	0.38	0.20	0.42	0.35

Combinations of EDAC and PHMPS

Preincubn Condn	Total ATPase	Mg^{2+}-ATPase	Total ATPase	Δ (Control − Inhibited Sample) Calcd[d] Total ATPase	Δ (Control − Inhibited Sample) Mg^{2+}-ATPase	Δ (Control − Inhibited Sample) Calcd[d] Mg^{2+}-ATPase
(a) 5 min with EDAC followed by 10 min with PHMPS	0.42	0.22	0.38	0.34	0.33	0.41
(b) 5 min with PHMPS followed by 10 min with EDAC	0.33	0.15	0.47	0.53	0.40	0.46

[a] All preincubations were carried at 23°. [b] Concentration of *p*-hydroxymercuriphenylsulfonic acid in preincubation mixture = 0.015 mM. [c] Concentration of EDAC in preincubation mixture = 0.500 mM. [d] Calculated on the basis of strict additivity from appropriate 5- and 10-min points with each inhibitor alone.

Hill plot of the Mg^{2+}-ATPase data in Table IV was linear with a slope of 1. As with EDAC, the presence of hydroxylamine protected substantially against the inhibitory effects of ECDQ.

Experiments to investigate the possibility of interaction between hydroxylamine and EDAC were performed. Hydroxylamine and EDAC (at the same concentrations as in the enzyme studies) were incubated for varying times prior to the addition of the enzyme preparation. No effect of such preincubation was seen on the level of enzyme inhibition—even when attempt was made to decrease the rate of such a reaction by carrying out the preincubation in ice. The protective effect of hydroxylamine would thus appear to be exerted at the level of the membrane–EDAC adduct.

On the basis of the modifying effects of nucleophile on the inactivation process and the identical qualitative behavior of EDAC and ECDQ, a carboxyl group appeared likely to be the common site of interaction of both inhibitors with the erythrocyte membrane.

CROSS-LINKING OF ACTIVATED CARBOXYLS WITH MEMBRANE NUCLEOPHILES. The finding that nucleophiles protected against EDAC attack was consistent with the susceptibility of the intermediate carbodiimide complex to nucleophilic attack and raised the hypothesis of an intramolecular cross-link between the activated carboxyl and a neighboring nucleophilic group on the membrane. The most likely candidates for this nucleophile component appeared to be a sulfhydryl, a hydroxyl, or an amino group.

Membrane sulfhydryl groups were blocked with *p*-hydroxymercuriphenylsulfonic acid in order to determine if this treatment interfered with the susceptibility of the membrane to EDAC attack. The results are shown in Table V, and the apparent additivity suggested independent sites of action of the two classes of inhibitors.

With regard to the possibility of a hydroxyl group acting as a nucleophile in the inhibition process, the resulting ester linkage might be susceptible to hydroxylamine attack. The complete insensitivity of the already inhibited enzyme to hydroxylamine reversal under the present experimental conditions would tend to cast some doubt on the possibility that the proposed cross-link is an ester bond.

The last alternative to be considered was that the cross-link is a peptide bond. Membranes were treated with reagents known to modify membrane amino groups in order to examine whether a decreased susceptibility to EDAC inhibition occurred. Succinic anhydride treatment resulted in partial or complete solubilization of the membrane, with complete loss of ATPase activity. Limited dinitrophenylation of the membrane with 2,4-dinitrofluorobenzene caused little or no alteration in ATPase activity or in the EDAC susceptibility, while more extensive treatment resulted in disruption of the membrane structure with subsequent solubilization. Therefore, the critical experiment could not be performed.

NUCLEOTIDE PROTECTION. In order to examine the possible relationship between the site of attack of EDAC and the active site of the membrane ATPase, the possibility of substrate protection by ATP against carbodiimide inactivation was investigated. ATP was found to afford almost complete protection at a concentration of 2 mM (Figure 2). While AMP was virtually ineffective in reducing the inhibitory effects of EDAC, ADP had an intermediate effect. However, when other purine and pyrimidine nucleotide di- and triphosphates were tested, they were found to exhibit the same behavior as the corresponding adenine nucleotide. Further, these nucleotide effects did not require the presence of Mg^{2+}.

Characterization of Carbodiimide-Modified ATPase. The basic point to be established here was whether EDAC attack

TABLE VI: Kinetic Parameters of Native and EDAC-Treated ATPase.[a]

		$K_{m,app}$ ATP[b] (mM)	V_{max} (μmoles of P_i/mg of Protein per hr)
Control membranes	Total ATPase	0.28	1.10
	Mg^{2+}-ATPase	0.17	0.48
EDAC-treated membranes	Total ATPase	0.28	0.62
	Mg^{2+}-ATPase	0.17	0.28
(EDAC + NH_2OH)-treated membranes	Total ATPase	0.22	0.91
	Mg^{2+}-ATPase	0.15	0.43

[a] Preincubation conditions: 5 min, 23°. Concentration EDAC in preincubation: 2.2×10^{-3} M. Concentration NH_2OH in preincubation: 2.0×10^{-3} M. [b] Concentration range of ATP: 0.05–2.0 mM.

on the membranes resulted in complete inactivation of the enzyme in an all or none manner or whether carbodiimide attack generated a modified enzyme with altered catalytic properties.

KINETIC PARAMETERS. The kinetic parameters, $K_{m,app}$ for ATP and maximal velocity of ATP hydrolysis (V_{max}) for native, EDAC-treated, and EDAC–hydroxylamine-modified membranes were determined for total and Mg^{2+}-ATPase activities (Table VI). EDAC treatment did not alter the $K_{m,app}$ for ATP, the values being 0.3 and 0.2 mM for total and Mg^{2+}-ATPase, respectively. Carbodiimide treatment did lead to a decrease in V_{max} from 1.10 to 0.62 μmoles of P_i per mg per hr for total ATPase and from 0.48 to 0.28 μmole of P_i per mg per hr for Mg^{2+}-ATPase. The activity of the EDAC–hydroxylamine-modified enzyme was indistinguishable from that of the control (untreated) enzyme.

pH–ACTIVITY PROFILES. The effect of the carbodiimide on the pH profile for catalysis of ATP hydrolysis was determined (Figure 3). The data were expressed on a relative basis with the maximal activity (at pH 7.5 in all cases) being arbitrarily set at 100. No perturbation of the ATPase pH–activity profile was detectable on EDAC treatment.

NUCLEOPHILE MODIFICATION OF EDAC-ACTIVATED CARBOXYL. In an attempt to generate enzymes with modified ATPase activities and possibly altered cation activation patterns, membranes were treated with EDAC in the presence of amino acid esters with side chains containing potentially charged groups of different sign. A negative charge would be substituted in the case of tyrosine methyl ester, and a positive charge for histidine methyl ester; glycine methyl ester was used as an internal (uncharged) standard. Evidence that these nucleophiles were indeed incorporated at the site of EDAC attack was obtained from [^{14}C]glycine ethyl ester (Table VII). As discussed below, results of studies indicated a carbodiimide-dependent incorporation of the labeled amino acid ester nucleophile into the lipid-free fraction of the membrane. None of these nucleophile derivatives of the membrane exhibited pH profiles significantly different from the native ATPase in the pH range from 6 to 9 (Figure 4).

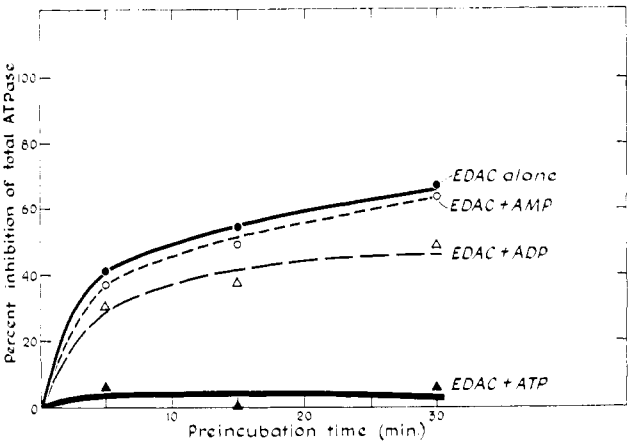

FIGURE 2: Inhibition of membrane total ATPase activity by EDAC alone and in the presence of AMP, ADP, and ATP. Concentration of EDAC in preincubation = 2.2 mM. Concentration of adenine nucleotide in preincubation = 2 mM.

Site of Carbodiimide Attack. Since carboxyl groups are present in the carbohydrate, lipid, and protein components of the erythrocyte membrane, it was of interest to determine which of these three components might be involved in EDAC attack. Erythrocyte membranes were treated with EDAC and [^{14}C]glycine ethyl ester, with resultant incorporation of the radioactive label at the site of carbodiimide attack. Membrane lipids were extracted with chloroform–methanol (2:1, v/v) leaving a lipid-free protein residue and a lipid extract. As may be seen from Table VIIa, EDAC was necessary for the incorporation of ^{14}C into the membrane, and negligible amounts of isotopic activity were found in the lipid fraction of the membrane.

Removal of the membrane sialic acids by neuraminidase treatment only slightly decreased the incorporation of labeled glycine ethyl ester into the membrane (Table VIIb), and the time course of EDAC inhibition of ATPase (Table VIII) was virtually unchanged.

Carbodiimide Specificity. EFFECT OF EDAC MODIFICATION ON OTHER MEMBRANE ENZYMES. The rather selective effects

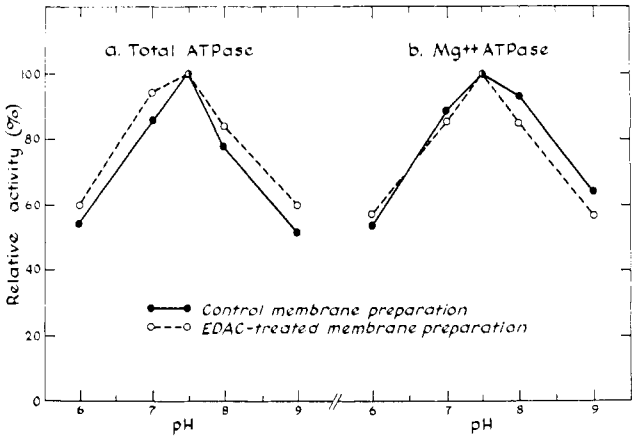

FIGURE 3: pH–activity profile of total ATPase (a) and Mg^{2+}-ATPase (b). Concentration of EDAC, 2.2×10^{-3} M in preincubation. Preincubation conditions: 5 min at 23°.

TABLE VII: Incorporation of [^{14}C]Glycine Ethyl Ester[a] into Erythrocyte Membranes.

	Whole Membranes		Lipid-Free Membranes		Lipid Extract	
	μmole of ^{14}C/mg of Protein		μmole of ^{14}C/mg of Protein		μmole of ^{14}C/mg of Lipid	
	−EDAC	+EDAC[b]	−EDAC	+EDAC	−EDAC	+EDAC
(a) Control membranes	0.006	0.052	0.003	0.059	0.0007	0.0006
(b) Neuraminidase-treated[c] membranes	0.007	0.048	0.003	0.039	0.0004	0.0008

[a] Concentration [^{14}C]glycine ethyl ester = 1.1×10^{-3} M. [b] Concentration EDAC = 0.8×10^{-3} M. Preincubation conditions: 30 min at 37°. [c] Details of neuraminidase treatment in Methods.

of EDAC on Mg^{2+}-ATPase activity suggested there was a degree of specificity in the EDAC-induced membrane modification. Thus, the effects of EDAC attack on other membrane enzymes were investigated (Table IX). In a membrane preparation in which ATPase activity was completely inhibited as a result of extensive EDAC treatment, there was no significant loss of DPNase activity of the membrane (Table IXb). Similarly, inhibition of ATPase by EDAC treatment did not affect the activity of nonspecific (K^+-independent) *p*-nitrophenyl phosphatase (Table IXa).

On the other hand, the activity of the K^+-dependent *p*-nitrophenyl phosphatase in the erythrocyte membrane was substantially decreased by the EDAC attack. Further study of the concentration dependence of this inhibition indicated a K_i value of 0.9 mM (Table Xa). Since it was of interest to study the interrelation between the ATPase and the K^+-stimulated *p*-nitrophenyl phosphatase systems, the behavior of both systems under identical preincubation conditions with EDAC and hydroxylamine was investigated (Table Xb). The K^+-dependent phosphatase was considerably less susceptible to carbodiimide inactivation than the ATPase in the presence of EDAC alone, and the phosphatase was also more susceptible to hydroxylamine protection from the inhibitory effects of the carbodiimide. Thus, although the two enzymes systems behaved in a qualitatively similar fashion, quantitative differences between the two were evident.

INCORPORATION OF [^{14}C]GLYCINE ETHYL ESTER AND EFFECT OF ADENINE NUCLEOTIDES. The object of these incorporation studies was to determine the proportion of membrane carboxyl groups susceptible to EDAC attack. Therefore, in order to achieve maximal labeling of the membrane protein, a higher concentration of EDAC (10 mM) was used in the incubation compared to that employed in typical inhibition experiments (1–2 mM). Further, in most of the inhibition experiments, preincubations of membranes with EDAC were usually no longer than 10–15 min as compared to 20 hr in the labeling studies. Thus it would appear reasonable to assume that the incorporation studies would yield an extreme upper limit for the proportion of those membrane carboxyls involved in EDAC attack and giving rise to ATPase inhibition. The extent of incorporation of labeled nucleophile (in the presence of EDAC) into the erythrocyte ghosts was followed as a function of time, and the results are summarized in Table XI.

The effects of ATP and AMP on the time course of incorporation of ^{14}C into the membranes were investigated (Table XII), and it was found that both nucleotides blocked incorporation, but ATP consistently exhibited a 20% greater decrease in incorporation as compared to AMP. These results contrasted somewhat with the effects of ATP and AMP on the EDAC-induced inactivation of ATPase, since

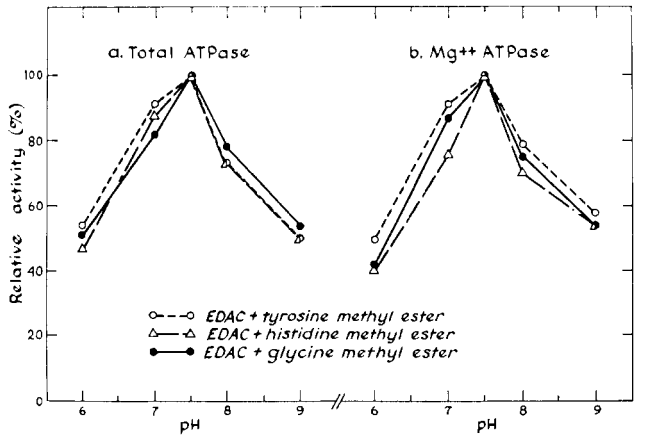

FIGURE 4: Modification of membrane total ATPase (a) and Mg^{2+}-ATPase (b) (by EDAC + amino acid ester nucleophiles, pH–activity profiles. Concentration EDAC in preincubation, 2.2×10^{-3} M. Concentration nucleophile, 2.0×10^{-3} M. Preincubation conditions, 5 min at 23°.

TABLE VIII: Effect of Prior Neuraminidase[a] Treatment on EDAC[b] Inhibition of Membrane ATPase.

Preincubn Time[c]	% Inhibn of Total ATPase	
	Untreated	Neuraminidase
(min)		
5	30	23
15	46	48
30	62	50

[a] Details of neuraminidase treatment in Methods. [b] Concentration EDAC in preincubation: 2.2×10^{-3} M. [c] Preincubation carried out in ice.

TABLE IX: Effect of EDAC on Membrane Phosphatases and DPNase.

Part a

Concn of EDAC in Preincubn (mM)[a]	ATPase Act. (μmoles of P_i/mg per hr)			Phosphatase Act. (mμmoles of Nitrophenol/mg per hr)		
	Total ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase	Total Phosphatase	K^+-Independent Phosphatase	K^+-Dependent Phosphatase
0	1.52	1.10	0.42	107	57	50
2	0.50	0.29	0.21	92	57	35
% inhibition	67	74	50	14	0	30

Part b

Concn of EDAC in Preincubn (mM)[b]	ATPase Act. (μmoles of P_i/mg per hr)			DPNase Act. (μmoles of DPN/mg per hr)
	Total ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase	
0	0.37	0.24	0.13	0.87
5	0.00	0.00	0.00	0.95
% inhibition	100	100	100	

[a] Preincubation conditions: 10 min, 23°. [b] Preincubation conditions: 30 min, 23°.

ATP completely abolished the inhibitory effects of the carbodiimide, while AMP had no effect on the inactivation process.

Discussion

Incubation of hemoglobin-free human erythrocyte membranes with the water-soluble carbodiimide, EDAC resulted in inhibition of ATPase, the extent of which increased with time to 100% (Table Ia). At low levels of inhibition, the Mg^{2+}-ATPase component of total ATPase activity was preferentially inhibited, and stimulation of Na^+,K^+-ATPase was frequently apparent under these conditions (Table II), while with extensive inhibition, the preferential inactivation of the Mg^{2+}-ATPase disappeared. Since shearing of the membranes, with the resulting formation of microvesicles 0.6 μ or less in diameter (Schrier *et al.*, 1970) did not in any way alter the pattern of EDAC inactivation of ATPase, including the preferential attack on Mg^{2+}-ATPase (Tables Ib and IIb), the mechanism of carbodiimide inactivation did not appear to depend upon the gross structural features of the membrane or its permeability characteristics.

The analogous effects of the carbodiimide and the selective carboxyl group reagent ECDQ (Belleau and Maleck, 1968; Belleau *et al.*, 1968) on ATPase activity suggested that the observed inhibition resulted from modification of membrane carboxyls (Table IV). Furthermore, the protective effects of nucleophiles (hydroxylamine and amino acid methyl esters) against enzyme inactivation when added simultaneously with EDAC were consistent with a competition between the added nucleophile and a membrane nucleophile for attack on the reactive carbodiimide–carboxyl group adduct. Carbodiimide inactivation of ATPase was interpreted in terms of a distortion induced in the membrane as a result of cross-link formation between an EDAC-activated carboxyl and a suitably oriented membrane nucleophile.

The nucleophilic component of the proposed intramolecular cross-link could be a sulfhydryl, a hydroxyl, or an amino group. The additivity of ATPase inhibition by *p*-hydroxyphenylmercurisulfonic acid and EDAC (Table V) made a sulfhydryl group an unlikely candidate. The insensitivity of the inhibited enzyme to hydroxylamine regeneration would tend to argue against the involvement of a hydroxyl group. A number of ester linkages are susceptible to hydroxamate formation under the relatively mild conditions employed in these studies and this should include protein-bound ester linkages. It might be mentioned, however, that the susceptibility of collagen to hydroxylaminolysis, formerly attributed to the existence of ester-like linkages within the protein, has recently been shown to be due to the presence of cyclic imide linkages (Bornsetin, 1970). On the basis of the hydroxylamine protection experiments (Table III) such a hydroxamate derivative, if formed, should have been fully active. However, the drastic conditions required for hydroxylaminolysis of some ester linkages would have resulted in complete loss of enzyme activity and hence could not be investigated. Experiments devised to test whether the proposed nucleophile moiety was indeed an amino group were unsuccessful, since succinic anhydride and 2,4-dinitrofluorobenzene caused generalized disruption of the membrane.

EDAC attack on the membrane probably resulted in complete inactivation of ATPase rather than in the formation of a modified enzyme with decreased catalytic activity for the following reasons: carbodiimide attack led to a decrease in V_{max} for ATP hydrolysis, but had no effect on $K_{m,app}$ for ATP (Table VI), and furthermore, the pH–activity profiles for catalysis of ATP hydrolysis by native and EDAC-treated membranes were identical in the range of pH from 6 to 9 (Figure 3).

The relationship of the site of EDAC attack to the active site of the membrane ATPase system was investigated by studying the effects of nucleophile modification on the membrane or enzyme carboxyl group presumed to be the

TABLE X: Concentration Dependence of K^+-Dependent *p*-Nitrophenyl Phosphatase by EDAC.

Concn of EDAC in Preincubn Mixture[a] (mM)	Part a μmole of *p*-Nitrophenol/mg of Protein per hr	% Inhibn of K^+-Dependent Phosphatase
0	0.045	0
1.0	0.024	47
2.0	0.016	64
4.0	0.007	84
6.0	0.009	81
8.0	0.002	94
10.0	0.003	94
	$K_i = 0.9 \times 10^{-3}$ M	

Concn of NH_2OH in Preincubn Mixture[b] (mM)	Part b % Inhibn of Na^+,K^+-ATPase	% Inhibn of K^+-Dependent Phosphatase
0	77	42
1.0	58	16
2.0	31	0
5.0	10	0

[a] Preincubated 15 min at 25°. [b] 10 min at 25°, concentration EDAC = 2.0×10^{-3} M.

site of EDAC attack. The formation of a membrane carboxyl hydroxamate could not be directly demonstrated because isotopically labeled hydroxylamine was not available. However, since incorporation of [^{14}C]glycine ethyl ester into the membrane was EDAC dependent (Table VII) and hydroxylamine was superior to all of the amino acid ester nucleophiles in protecting against EDAC attack, it appeared likely that a membrane hydroxamate was formed in the presence of EDAC and hydroxylamine. Since the postulated hydroxamate derivative exhibited behavior indistinguishable from that of the native enzyme (Table VI), it is unlikely that the carboxyl

TABLE XI: Time Course of Incorporation of [^{14}C]Glycine Ethyl Ester into Erythrocyte Membranes in the Presence of EDAC.[a]

Time (hr)	μmole of [^{14}C]GlyOEt Incorpd/mg of Membrane Protein
0.5	0.087
1	0.120
5	0.160
20	0.200

[a] Concentration of EDAC in preincubation mixture; 10^{-2} M; concentration of [^{14}C]glycine ethyl ester in preincubation mixture, 2×10^{-2} M.

TABLE XII: Effect of Nucleotides on EDAC-Dependent Incorporation of [^{14}C]Glycine Ethyl Ester into Erythrocyte Membranes.[a]

Sample	Preincubn Time (min)	mμmoles of [^{14}C]GlyOEt Incorpd/mg of Protein	% Incorp Rel to Control
Control	5	0.76	100
	15	1.27	100
	30	1.69	100
ATP (3 mM)	5	0.31	41
	15	0.48	38
	30	0.62	36
AMP (3 mM)	5	0.44	58
	15	0.75	59
	30	0.95	56

[a] Preincubation conditions: concentration EDAC 1.8×10^{-3} M; concentration [^{14}C]glycine ethyl ester = 1.6×10^{-4} M (temperature, 23°).

involved in EDAC inactivation of ATPase is the active-site γ-glutamylcarboxyl group proposed to be involved in the Na^+-dependent formation of the acyl-phosphate intermediate in ATP hydrolysis (Hokin *et al.*, 1965).

The introduction of ionizable groups such as the phenolate ion of tyrosine methyl ester or the imidazolium ion of histidine methyl ester did not perturb the pH–activity profiles of the ATPase activity of the membrane (Figure 4), thereby providing further evidence that the site of EDAC attack on the membrane was not in the immediate vicinity of the active site of the ATPase.

Carbodiimide attack could have inactivated ATPase either by inducing a generalized disruption of the membrane, or by selectively altering some component relevant to the ATPase system. A selective interaction at the level of the ATPase system would be expected to have little effect on other membrane enzymes. Extensive EDAC attack was found to have no effect on the activity of the K^+-independent (nonspecific) *p*-nitrophenyl phosphatase (Table IXa) or of DPNase (Table IXb), an enzyme located on the outer surface of the membrane (Gitler *et al.*, 1967) which has been used as an indication of erythrocyte membrane integrity.

However, inhibition of the K^+-dependent *p*-nitrophenyl phosphatase was observed (Table Xa). It has been proposed that this activity might represent the terminal dephosphorylation stage of ATP hydrolysis by Na^+,K^+-ATPase (Albers, 1967). It was therefore significant that both Na^+,K^+-ATPase and K^+-dependent *p*-nitrophenyl phosphatase were susceptible to EDAC modification (Table IXa) although the phosphatase was less susceptible to carbodiimide inactivation and more susceptible to hydroxylamine protection (Table Xb). This observation could reflect a preferential effect of EDAC on the Na^+-dependent phosphorylation in ATP hydrolysis by ATPase.

The possible role of membrane conformation in the carbodi-

imide inactivation of ATPase was suggested by the protective effects of nucleotide di- and triphosphates on EDAC inhibition (Figure 2). These effects were presumably not due to direct interaction at the active site of ATPase, since nonsubstrate purine and pyrimidine nucleotide di- and triphosphates exhibited behavior identical with that of the corresponding adenine nucleotide. It has been reported that nucleotides modify membrane conformation (Askari and Koyal, 1968). In particular, our results appear to parallel the findings of Hoffman and Ingram (1968) who found that ATP (and other nucleotide triphosphates) promoted the binding of tritiated ouabain to erythrocyte ghosts; ADP exhibited an intermediate effect, while no binding was observed with AMP.

Under conditions for maximal binding of labeled nucleophile to the protein component of the membrane 0.2 μmole of glycine [^{14}C]ethyl ester was incorporated per mg of membrane protein (Table XI), whereas it was calculated that each mg of membrane protein contained 1.85 μmoles of acidic amino acid carboxyl groups (Rosenberg and Guidotti, 1968). In the inhibition experiments, concentrations of EDAC four times lower than in the labeling studies gave rise to total inhibition after only 1 hr. These considerations emphasize the limited extent of carbodiimide reaction giving rise to total inhibition of the membrane ATPase.

The effects of nucleotides on the incorporation of [^{14}C]-glycine ethyl ester into membranes were investigated. Despite the fact that AMP exhibited negligible protection against carbodiimide inactivation of ATPase, it substantially decreased the incorporation of [^{14}C]glycine ethyl ester in the presence of EDAC (Table XII). The difference in labeled glycine ethyl ester incorporation in membranes preincubated with ATP and AMP may reflect precisely those carboxyls which are directly relevant to the EDAC-induced inhibition of ATPase.

Carbodiimide attack, therefore, does not result in widespread membrane disruption, nor does it appear to affect carboxyls at or adjacent to the active sites of ATPase. The carbodiimide inhibition appears to involve very limited activation of membrane protein carboxyls, followed by intramolecular cross-linking with proximal membrane nucleophiles, most likely amino groups. This cross-linking would cause perturbation of the membrane structure, resulting in inactivation of the ATP-hydrolyzing system. The patterns of nucleotide protection both against EDAC-induced inhibition of ATPase, as well as EDAC-mediated reaction of protein carboxyls with labeled nucleophiles, are consistent with the concept that EDAC attack results in an alteration of membrane conformation. The observations of Abrams *et al.* (1969) on the carbodiimide-induced inhibition of a membrane bound ATPase, as well as a number of energy-dependent transport processes in *S. faecalis* membranes, and the finding that carbodiimides block an ATPase and oxidative phosphorylation at the inner mitochondrial membrane (Bulos and Racker, 1968) might well be rationalized within the mechanistic framework outlined here. It may be that the susceptibility of these membrane systems to carbodiimide attack and the similar biochemical consequences of such attack reflects a common structural or functional organization essential for the energy transduction properties of these membranes.

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